IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.	:	10/518,723	Confirmation No. 1009
Applicant	:	NICOLAAS DUNEAS	
Filed	:	August 10, 2005	
Title	:	METHOD OF PREPARING AN OSTEOGENIC PROTEIN FRACTION	
Group Art Unit	:	1646	
Examiner	:	Elizabeth C. Kemmerer	
Customer No.	:	28289	

Commissioner for Patents
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DECLARATION UNDER 37 C.F.R. §1.132

I, Nicolaas Duneas, declare that:

1. I received a PhD degree from the University of the Witwatersrand in South Africa in 1998.

2. I have read and understand the Office Actions dated April 28, 2008 and October 30, 2008 as well as the cited prior art. This Declaration is provided in response to the rejection under 35 U.S.C. §103(a) as being obvious over United States

patent number Scott et al. (1994, The Anatomical Record 238:23-30) (hereinafter "the Scott reference") or Yoshimura et al. (1993, Biol. Pharm. Bull. 16(5):444-447) (hereinafter "the Yoshimura reference") in view of United States Patent No. 4,968,590 to Kuberasampath et al. (hereinafter "the Kuberasampath patent".

3. I understand the invention of Scott to be directed towards a method of preparing an osteogenic protein fraction (see Abstract). The method includes extracting demineralised bone matrix with a solution of at least one chaotropic agent selected from the group consisting of urea and gaunidine salts (see left column of second page). The bone matrix proteins which are isolated have molecular weights of between 10 and 100kDa (see left column of second page). A 100 kDa nominal molecular weight ultrafiltration membrane is used by the invention of Scott, in contrast to the claimed invention, which teaches the use of a 300 kDa nominal molecular weight ultrafiltration membrane. The protein fraction is subjected to heparin affinity chromatography (see Abstract and left column of second page). The purified osteogenic protein fraction is then exchanged into a solvent suitable for human medical use.

4. I understand the invention of Yoshimura to be directed towards a method of preparing an osteogenic protein fraction (see title and Abstract). The method also includes extracting demineralised bone matrix with a solution of at least one chaotropic agent selected from the group consisting of urea and gaunidine salts (see Introduction, second paragraph). The bone matrix proteins which are isolated have molecular weights of between 10 and 100kDa (see Introduction, second last paragraph). A 100 kDa nominal molecular weight ultrafiltration membrane is used by Yoshimura, in contrast to the claimed invention, which teaches the use of a 300 kDa nominal molecular weight ultrafiltration membrane. The protein fraction is also subjected to heparin affinity chromatography (see Purification, step 3). The purified osteogenic protein fraction is then exchanged into a solvent suitable for human medical use.

5. I understand the invention of Kuberasampath also to be directed towards a method of preparing an osteogenic protein fraction (see Abstract and column

1, lines 35-37). The method also includes extracting demineralised bone matrix with a solution of at least one chaotropic agent selected from the group consisting of urea and gaunidine salts (see column 6, lines 32- 52). The bone matrix proteins which are isolated have molecular weights greater than 10kDa (see bottom of column 2 and top of column 3). A higher nominal molecular weight ultrafiltration membrane is not used by the invention of Kubersampath, in contrast to the claimed invention, which teaches the use of a 300 kDa nominal molecular weight ultrafiltration membrane. The protein fraction, which is therefore not depleted of collagen, is also subjected to heparin affinity chromatography (see column 6, lines 53-68 and column 7, lines 1-9). In addition, Kuberasampath subjects the heparin affinity chromatography fraction to hydroxyapatite chromatography (see column 7, lines 10-32). This step is not present in Scott or Yoshimura. The purified osteogenic protein fraction is then exchanged into a solvent suitable for human medical use.

6. I understand the claimed invention is directed to a method of preparing an osteogenic protein fraction, by extracting demineralized bone matrix with a solution of at least one chaotropic agent; removing high molecular weight proteins which exceed 300 kDA from the extract by ultrafiltration with a 300 kDA membrane to produce a lower molecular weight fraction; subjecting the lower molecular weight fraction to heparin affinity chromatography under conditions which first favor the binding and then the elution of a purified heparin affinity fraction containing the osteogenic protein fraction; subjecting the heparin affinity fraction to hydroxyapatite chromatography under conditions which first favor the binding and then the elution of a purified osteogenic protein fraction; and exchanging the purified osteogenic protein fraction into a solvent suitable for human medical use. The chaotropic agent is selected from the group consisting of urea and guanidinium salts to produce an extract.

7. The Examiner has rejected claim 36 under 35 U.S.C.103(a) as being unpatentable over either of Scott *et al.* or Yoshimura *et al.* in view of Kuberasampath *et al.* In particular, the Examiner has argued that the use of a 300 kDa membrane is obvious when isolating a small molecular weight protein and that the

higher yield will be at the expense of purity. On the face of this is arguably a logical assumption but, as described below, the assumption is not valid in commercial terms.

8. By way of a brief summary, in the process of the present invention, bone morphogenic protein (BMP-2) is isolated from demineralised bone matrix (DBM). In the process, proteins having molecular weights of more than 300 kDa are removed, under conditions of high ionic strength, at the beginning of the process using a 300 kDa ultrafiltration membrane. This is done prior to the chromatographic steps in order to improve the yields in chromatography steps as described below. The process is set out in Figure 1.

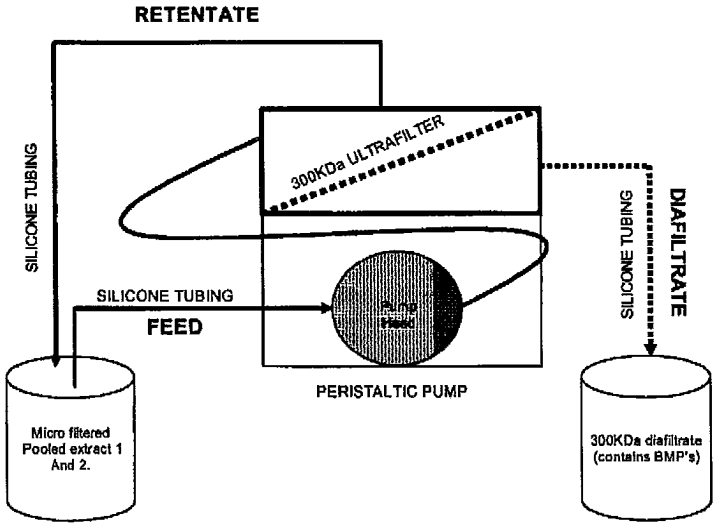

Figure 1. Ultrafiltration of DBM extract at high ionic strength conditions through 300 kDa membrane, to eliminate in the retentate, collagens and collagenous aggregates, and capture, in the diafiltrate, lower molecular weight matrix proteins, including BMP-2.

The applicant has found that contaminating collagens, and collagen aggregates in the intial extract from the demineralised bone matrix (DBM) interfere with the efficiency of the isolation of the bone morphogenetic proteins when using affinity chromatography.

The Applicant has found that the presence of high amounts of collagens and collagen aggregates typically co-extracted from DBM have a number of negative effects:

In the first place, high amounts of collagens and collagen aggregates in the initial extract cause fouling of chromatographic columns. This causes channeling phenomena, whereby proteins follow a path of least resistance through the affinity matrix, thereby bypassing binding sites on the chromatography media. In the second place, the Applicant has found that the protein BMP-2 binds demineralised bone matrix collagen at low ionic strength conditions. This is shown in Figures 2 and 3 below.

In the method of the present invention, collagens and collagen aggregates are separated from BMP-2 at the beginning of the process at conditions of high ionic strength, using either 1 M NaCl solutions or 4 M guanidinium chloride solutions, prior to exchange in low ionic strength media (6M urea, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) and then applied onto heparin affinity chromatography media. The Applicant has found that the competitive binding which takes place between BMP-2 and collagen, as demonstrated in Figure 2, is at least partly if not entirely responsible for BMP-2 losses during affinity chromatography on heparin chromatography media.

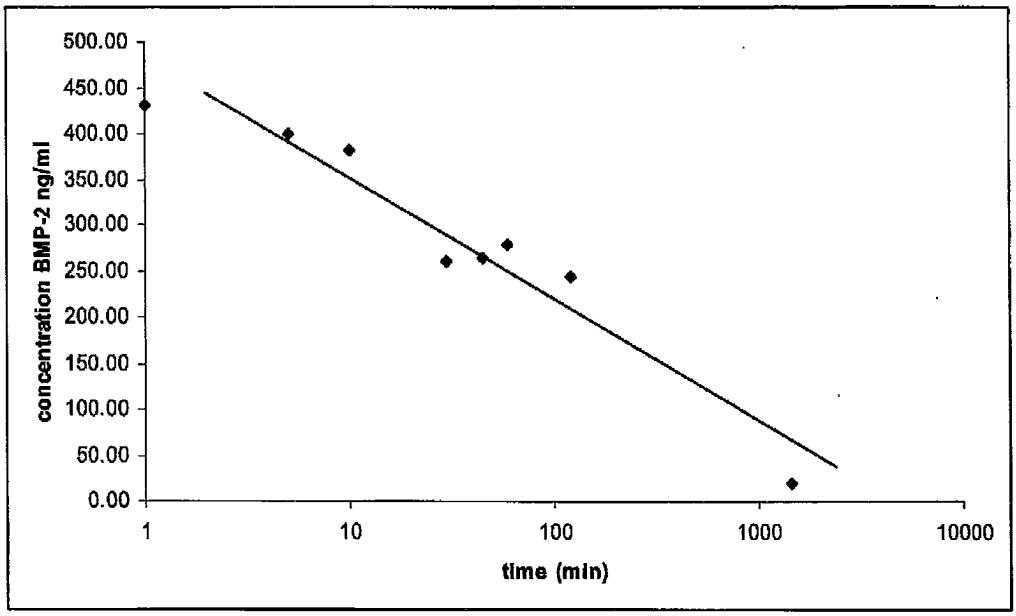

Figure 2. Binding of pBMP-2 to porcine bone matrix collagen over time (24 hours).

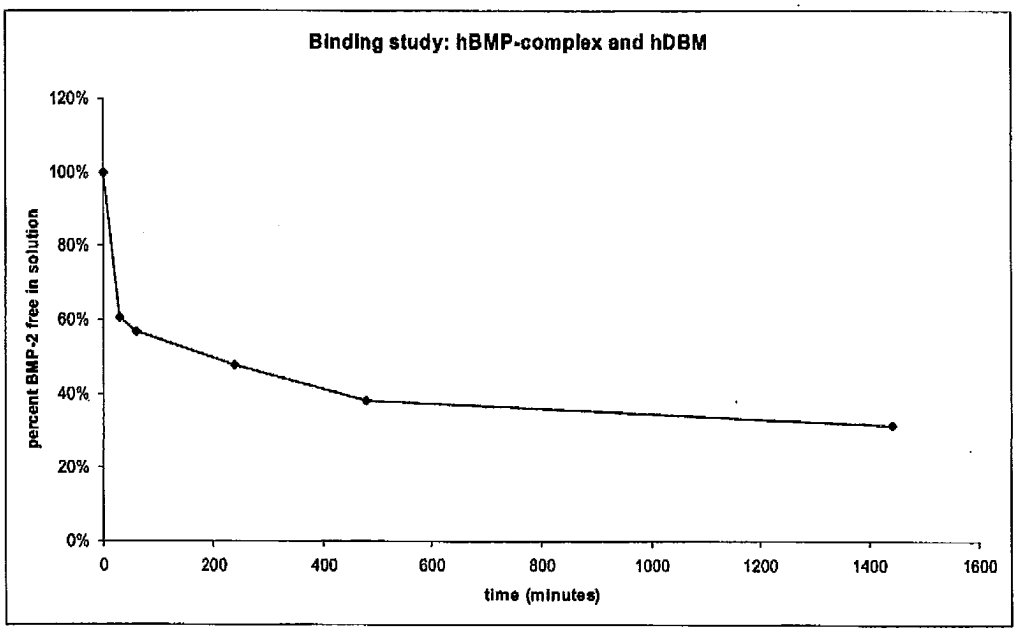

Figure 3. Binding of hBMP-2 to human demineralised bone matrix over time.

In order to show that the method of the present invention produces a higher yield of pure BMP-2 than the methods of Scott, Yoshimura and Kuberasampath, the Applicant has now conducted side-by-side laboratory trials using a 100 kDa membrane and a 300 kDa membrane.

Initially, to exclude column channeling effects, small samples of extracts were run through the chromatography columns, to avoid channeling phenomena which occur when full batches of extract are loaded. No visible fouling or channeling phenomena were observed in any of the experiments described below, and the yield improvements demonstrated by these experiments are, as discussed below, due to the alleviation of BMP-2 interacting with collagen.

In the laboratory trials Porcine demineralised bone matrix was prepared according to the method of Kuberasampath. Total proteins were extracted with 4M guanidinium chloride, 50 mM Tris-HCl, pH 7.4, and microfiltered through a 3 micron filter (Millipore 10" Polygard™), to remove large insoluble matrix particles. Soluble collagen and

smaller suspended aggregates were not removed in this step, and remained within the extract. The extract was then processed further into three experiments. The three fractions that were processed from the initial extract, were labeled as follows:

1. **crude extract**; initial extract was exchanged on a 5 kDa polyethersulfone membrane (catalogue number CDU F00 1TH; Millipore Corporation, USA).
2. **300 kDa fraction**; initial extract was ultrafiltered through a 300 kDa polyethersulfone membrane (catalogue # CDU F00 6TM; Millipore Corporation, USA), according to the method of the present invention, then exchanged into 6M Urea, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, on a 5 kDa polyethersulfone membrane (Millipore Corporation, USA)
3. **100 kDa fraction**; initial extract was ultrafiltered through a 100 kDa polyethersulfone membrane, then exchanged into 6M Urea, 150 mM NaCl, Tris-HCl pH 7.4,on a 5 kDa polyethersulfone membrane (catalogue number CDU F00 1TH; Millipore Corporation, USA).

A chromatography column (XK 50, General Electric Health Sciences, USA) was packed with 225 ml Prosep heparin affinity matrix (General Electric Health Sciences, USA) and equilibrated with 6M urea, 150 mM NaCl, Tris-HCl, pH 7.4.

Each of the three fractions were chromatographed on the Prosep column, eluting the peak fraction containing BMP-2 (Figure 3), using 6M Urea, 500 mM NaCl, pH 7.4. The peaks eluting at 118.81min, 118.41 min and 121.31 min (in chromatograms A, B and C respectively) in Figure 4 were the BMP complex peaks.

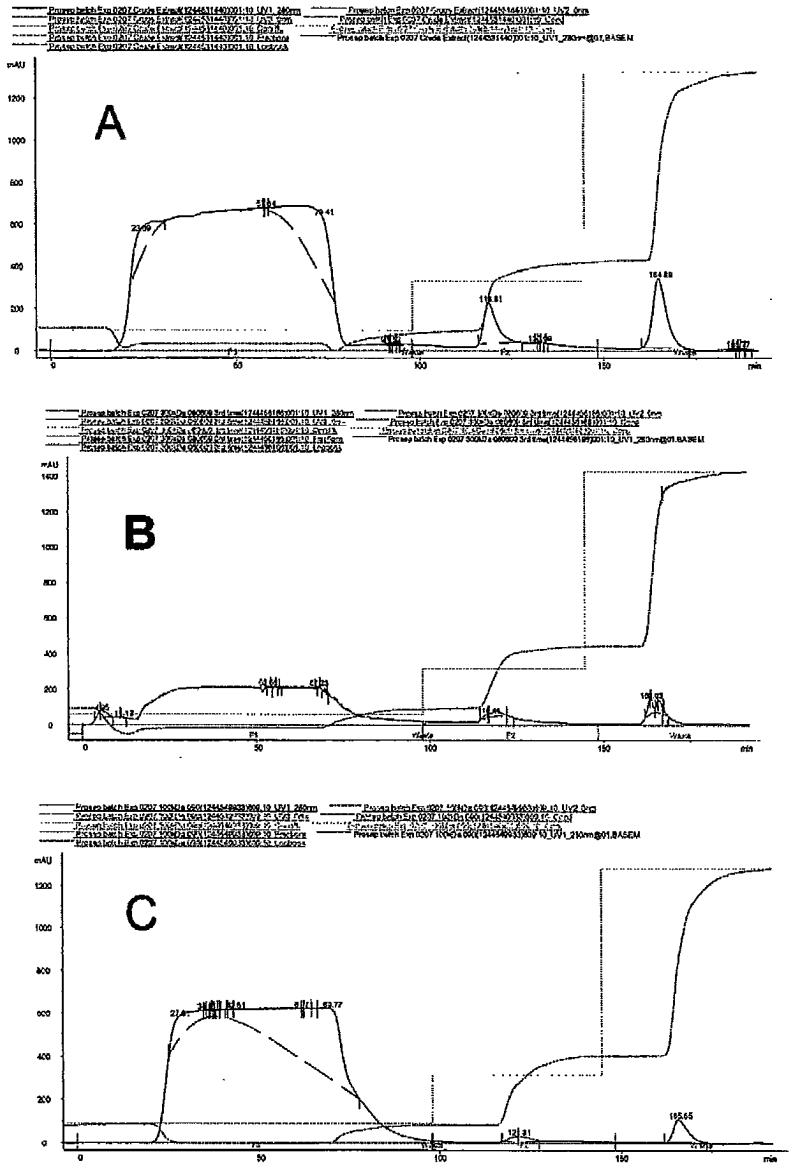

Figure 4. Chromatograms of porcine DBM extract on Prosep heparin affinity media. A. Method of Sampath et al., 1987. B. Method of present invention. C. Method of 100 kDa ultrafiltration.

A commercially available BMP-2 ELISA kit (Quantikine, R&D Systems, USA) was used to quantify the amount of BMP in each peak. The 300 kDA process yielded almost twice the amount of BMP-2 when compared to the 100 kDA procedure.

Table 1. BMP-2 yield normalized against total protein for each experiment.

	Amount BMP-2 (ng)	normalised fraction mAu	**Abundance BMP-2 ng/mAu**	**Relative performance of claimed invention versus other methods**
crude affinity fraction	29644.53	27,545	1.08	54%
300 kDa affinity fraction	54703.93	26,829	2.04	
100 kDa affinity fraction	30238.64	5,934 (22% of 300kDa	5.10	55%

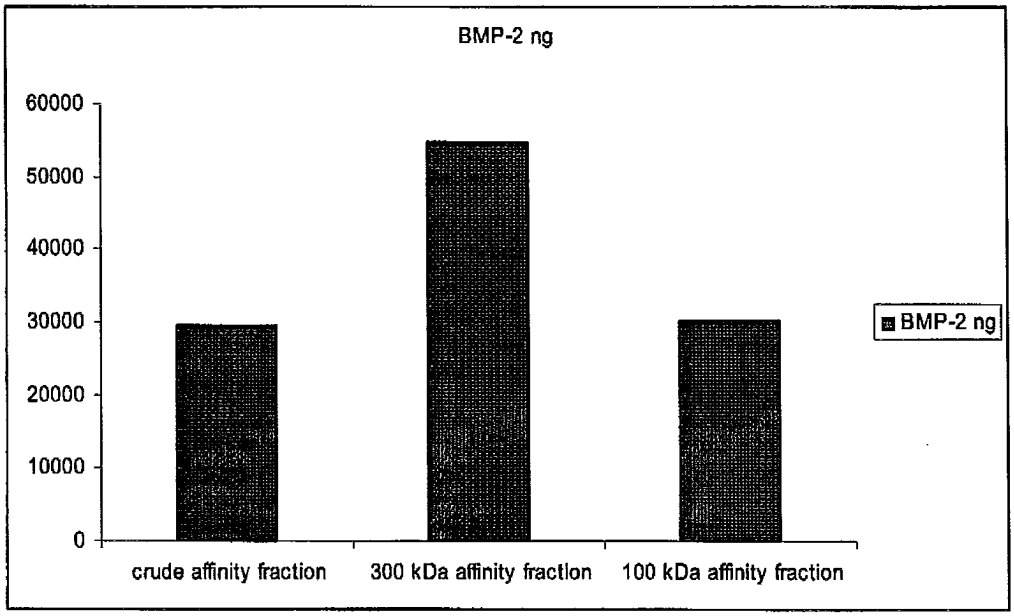

Figure 5. Results of BMP-2 isolation.

Almost twice the amount of BMP-2 was isolated in the 300 kDa fraction when compared to the crude affinity fraction and the 100 kDa fraction. These results exclude fouling and channeling effects which occur when running full production batches. In the Applicant's experience, the additional channeling effects created by collagen aggregates can further reduce the yield by a factor of two to four times. The use of the 100 kDa membrane clearly shows that total protein recovery in the affinity fraction is poor, only 22% in comparison to the 300 kDa membrane. The 100 kDa process produced only 55% of the amount of BMP-2 produced by the 300 kDa process. The BMP-2 abundance (concentration) was highest in the 100 kDa method (5.1 ng/mAu versus 2.04 ng/mAu for 300 kDa method), but in commercial terms, the 300 kDa method will produce double the amount of implantable devices, as each device is dosed as a function of an amount of BMP-2 per implantable device. In addition, the additional matrix proteins form part of the cocktail of morphogens which act synchronously and synergistically to improve bone formation, as exemplified by the synergistic interaction of transforming growth factor-beta and bone morphogenetic protein-7 in published studies using non-human primates (DUNEAS N, CROOKS J, RIPAMONTI U. 1997. Transforming Growth Factor-β1: Induction of bone morphogenetic genes expression during endochondral bone formation in the baboon, and synergistic interaction with Osteogenic Protein-1. *Growth Factors Vol 15; pp. 259-277.).* In this respect, the claimed invention allows for the high yield co-purification of the bone matrix derived morphogens transforming growth factor-beta and bone morphogenetic protein-7 on the heparin-Prosep column (Figure 6).

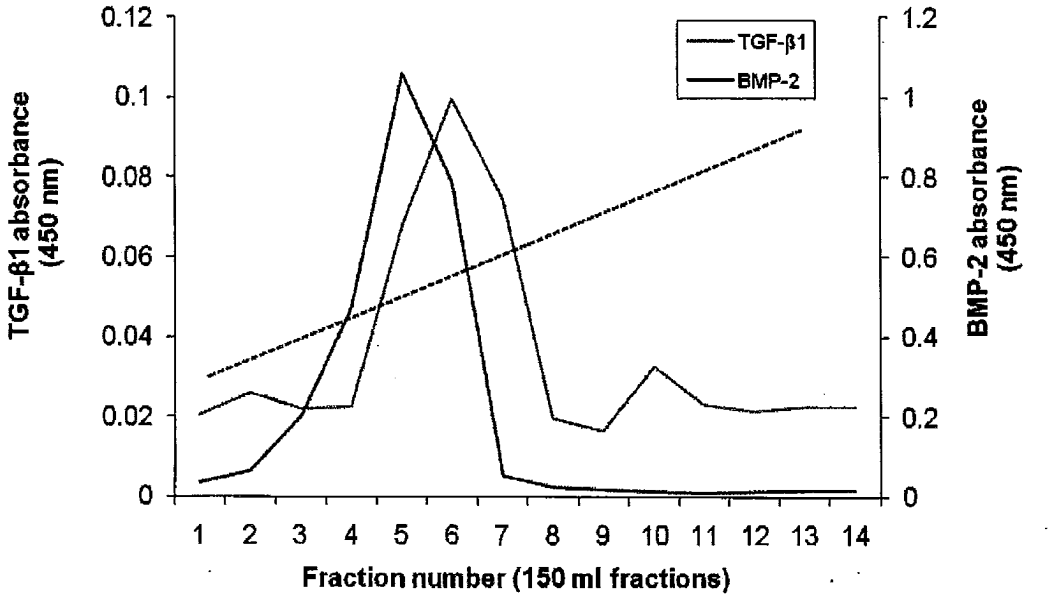

Figure 6. Elution of porcine TGF-β1 and BMP-2 on heparin-Prosep medium determined by ELISA assays

The method of Kubersampath (patent 4, 968,590, page 6 lines 35-65) relies on ethanol precipitation of total protein, at the step prior to heparin Sepharose chromatography. Ethanol precipitation with seven volumes of absolute ethanol will precipitate collagens and these will then be present during the next step of the Kubersampath process, which is heparin-Sepharose chromatography. In addition, presumably in order to overcome column fouling and channeling phenomena, all associated with the negative effects of collagen, the method of Kubersamath relies on the unpacking of the heparin-Sepharose column each time the procedure is performed, in order to mix the extracted proteins and the chromatographic media together before repacking the media into a column for subsequent chromatographic elution of the heparin binding proteins. The claimed invention does not suffer from these drawbacks, and consequently chromatography can be preformed normally, without the need to unpack and repack the chromatographic media each time.

The comparative study clearly shows that the method of the invention is more effective than the methods of the cited prior art. The Examiner's view that the larger pore size would be expected to produce a higher yield of the BMP-2 protein, but that this would be at the expense of a reduction in purity is accordingly not supported by the commercial benefits of a higher total yield of BMP-2, within a milieu of a synergistic cocktail of multiple growth factors as demonstrated by Figure 6. The Applicant accordingly submits that the use of a 300 kDa membrane to isolate a protein with a mass of 30-40 instead of a 100 kDa membrane produces a surprising increase in the yield of the pure protein. This choice was based on the Applicant's careful study of the processes which take place during the separation steps. The Applicant further submits that it would not have been obvious to a person skilled in the art to use a 300 kDa membrane if the derived protein BMP-2 has a mass of 30-40 kDa. The Applicant accordingly submits that the claimed invention is both new and non-obvious.

9. I declare under penalty of perjury under the laws of the United States that all statements made herein based on my own knowledge are true, and all statements made on information and belief are believed to be true. I acknowledge that willful false statements and the like are punishable by fine or imprisonment, or both and may jeopardize the validity of the application of any patent issuing thereon.

Nicolaas Duneas 25 November 2009

Declarant Date